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Kinetochore Localization of Murine Bub1 Is Required for Normal Mitotic Timing and Checkpoint Response to Spindle Damage

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Summary

The mitotic checkpoint ensures proper chromosome segregation by delaying anaphase until chromosomes are aligned on the spindle. Following prolonged spindle damage, however, cells eventually exit mitosis and undergo apoptosis. We show here that a murine homolog of the yeast mitotic checkpoint gene *BUB1* localizes to the kinetochore during mitosis. By expressing a dominant-negative mutant, we show that mBub1 is not only required for the checkpoint response to spindle damage, but acts in the timing of a normal mitosis. In addition, when mBub1 function is compromised, cells escape apoptosis and continue cell cycle progression, despite leaving mitosis with a disrupted spindle. These data demonstrate a role for kinetochore-associated mBub1 in regulating exit from mitosis, and suggest functional links between the mitotic checkpoint and subsequent apoptotic events in G1.

Introduction

The transmission of genetic information relies on checkpoint responses to errors concerning the integrity, replication, and segregation of the genome (Hartwell and Weinert, 1989; Murray, 1992; Hartwell and Kastan, 1994; Elledge, 1996). The mitotic checkpoint delays the onset of anaphase until chromosomes are aligned on the spindle (reviewed in Murray, 1994, 1995; Gorbsky, 1995; Rudner and Murray, 1996; Wells, 1996; Nicklas, 1997). However, following prolonged arrest in response to spindle damage, cells exit mitosis, return to interphase, and then either enter additional cell cycles (Kung et al., 1990; Andreassen and Margolis, 1994) or die by apoptosis (Minn et al., 1996). The signals that activate apoptosis following an abortive mitosis are not known.

Recently, it has been shown that the mitotic checkpoint is activated by the presence of unattached kinetochores (Rieder et al., 1994; Rieder et al., 1995). While some evidence suggests that the lack of microtubule attachment directly activates the checkpoint (Rieder et al., 1995), other observations indicate that the checkpoint monitors tension at the kinetochore (Li and Nicklas, 1995; Nicklas et al., 1995). These latter observations support an earlier proposal that tension-sensitive enzymes at the kinetochore generate diffusable anaphase inhibitors (McIntosh, 1991). Upon attachment to spindle microtubules, these kinetochore enzymes become inactive, the signals inhibiting anaphase dissipate, and chromosome segregation proceeds. Additional evidence for this hypothesis came from observations made with the 3F3 anti-phosphoepitope antibody, which specifically recognizes kinetochores that are not attached to the spindle (Gorbsky and Ricketts, 1993; Campbell and Gorbsky, 1995). In grasshopper spermatocytes, kinetochores not under tension stain brightly with the 3F3 antibody. However, when tension is applied, either from natural mitotic forces or artificially by a micromanipulation needle, 3F3 staining is diminished (Nicklas et al., 1995). One interpretation of these observations is that anaphase is inhibited by the activity of a tension-sensitive kinase located at the kinetochore. The identity of this kinase, and how its phosphorylated substrate might inhibit anaphase, are unknown.

Genetic screens have identified several yeast genes required for pre-anaphase delay in response to spindle disruption. These include *BUB1*, *2*, and *3* (Hoyt et al., 1991), and *MAD1*, *2*, and *3* (Li and Murray, 1991). Bub1 is a protein kinase that can bind and phosphorylate Bub3 (Roberts et al., 1994). Mad1 can bind Mad2 and becomes phosphorylated when spindle assembly is disrupted (Hardwick and Murray, 1995). Mps1, a protein kinase required for spindle pole body duplication and mitotic checkpoint function (Weiss and Winey, 1996), appears to phosphorylate Mad1 directly (Hardwick et al., 1996). A combination of genetics and biochemistry based on Mad1 phosphorylation has defined a signal transduction cascade that is activated in response to spindle damage and results in cell cycle arrest. This analysis places Bub1, Bub3, and Mps1 upstream of Mad1 and Mad2, while Bub2 and Mad3 appear to act downstream of these genes (Hardwick and Murray, 1995; Elledge, 1996; Hardwick et al., 1996; Rudner and Murray, 1996; Wells, 1996). Significantly, recent evidence suggests that this pathway responds to defects in kinetochore and centromere structure (Wang and Burke, 1995; Pangilinan and Spencer, 1996; Wells and Murray, 1996). We therefore speculated that these proteins might play a role in the kinetochore-mediated mitotic checkpoint described in higher eukaryotes. Indeed, vertebrate homologs of Mad2 have recently been shown to localize to the kinetochore (Chen et al., 1996; Li and Benezra, 1996), and Xenopus Mad2 is required to maintain MPF activity in an in vitro system that reconstitutes the mitotic checkpoint (Chen et al., 1996).

Here, we describe the functional analysis of a murine homolog of *BUB1*. We show that mBub1 localizes to the kinetochore and that this localization is essential for both checkpoint function in response to spindle damage and the timing of normal mitotic exit. Finally, we show that when the mBub1-dependent checkpoint is compromised, cells bypass the apoptotic response that normally occurs when cells override an activated checkpoint.

Results

Structural Comparison of Bub1 Homologs

A screen of expressed sequence tag (EST) databases with yeast spindle assembly checkpoint proteins identified a partial mouse cDNA (Kerr et al., 1994) with homology to the Saccharomyces cerevisiae protein kinase

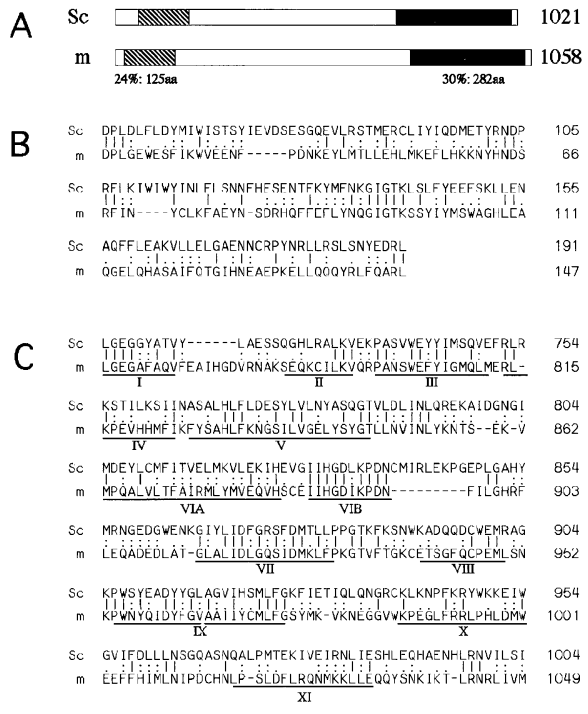

Figure 1. Homology between mBub1 and ScBub1

(A) Schematic representation of Bub1 homologs. The hatched box represents the 125 amino acid N-terminal homology domain, and the closed box represents the homologous C-terminal, catalytic domain of these kinases. No homology was detected in the domain intervening the N- and C-terminal domains.

(B) Sequence comparison of the N-terminal homology domain. Vertical bars indicate conserved residues, while double and single dots represent conservative and semiconservative substitutions, respectively.

(C) Sequence comparison of the C-terminal kinase domain. The 12 subdomains conserved among kinases are underlined.

Bub1 (ScBub1) (Roberts et al., 1994). We isolated a corresponding full-length murine cDNA with a 3174 bp open reading frame that encodes a 1058 amino acid polypeptide with a predicted molecular mass of 119.5 kDa. This protein, mBub1, has two domains homologous to ScBub1 (Figure 1). The 125 amino acid N-terminal domain shares 24% identity and 51% similarity with the N terminus of ScBub1 (Figures 1A and 1B), while a 282 amino acid region at the C terminus shares 30% identity and 60% similarity (Figures 1A and 1C) with the yeast protein. This C-terminal region corresponds to the kinase domain of ScBub1 and is similar to the catalytic domains of other protein kinases (Hanks and Hunter, 1995; Figure 1C). The central domain shows no significant homology to ScBub1 or other proteins on the NCBI database.

mBub1 Localizes to the Kinetochore during the Early Stages of Mitosis

To determine the subcellular localization of mBub1, we generated monoclonal antibodies against a portion of the protein (amino acids 332–731). Monoclonal antibodies from two independent hybridomas were characterized in detail and yielded identical results. Data obtained with one of these, 4B12, are presented here. By immunoblotting, this antibody recognizes the antigen when overexpressed in yeast and detects a single species in mouse protein extracts at the molecular weight expected of mBub1 (not shown). Hybridoma tissue culture supernatant was used to localize mBub1 in dividing mouse fibroblasts (Figure 2). Interphase and mitotic kinetochores were labeled with autoantibodies from sera of patients with the CREST syndrome of scleroderma (Moroi et al., 1980). In interphase cells, 4B12 staining was variable (not shown). When detectable, staining was observed diffusely throughout the nucleus, excluded from the nucleoli, and often concentrated in heterogeneous foci that did not appear to colocalize with CREST antigens. Paired foci of CREST staining were usually visible in interphase cells that stained with 4B12, suggesting that these cells were in late S phase or G2. During prophase, as judged by chromatin condensation, 4B12 staining appeared as pairs of foci that localized with CREST antigens (Figure 2A). Some diffuse nuclear staining was also present. In cells bearing a prometaphase rosette of chromosomes (Figure 2B), virtually all the 4B12 staining colocalized with CREST antigens. At metaphase, the CREST antigens were clearly associated with the chromatin along the metaphase plate (Figure 2C), while 4B12 appeared dispersed throughout the cell. During anaphase, 4B12 staining remained dispersed throughout the dividing cell (Figure 2D). Based on these observations, we conclude that murine Bub1 localizes to the kinetochore during prophase and prometaphase, but not during or after metaphase. When cells were treated with nocodazole to inhibit microtubule polymerization, kinetochores stained strongly with 4B12 (Figure 2E). Significantly, in untreated cells, kinetochores of lagging chromosomes stained strongly with 4B12, while aligned chromosomes showed only faint staining (Figure 2F).

Domain Analysis of mBub1

To determine which domain of mBub1 was responsible for kinetochore localization, we assayed various deletion mutants (Figure 3A) by transient transfection of BHK cells. Full-length mBub1 localized to the kinetochore during prophase (Figure 3B). In contrast to endogenous protein, however, ectopically expressed mBub1 was predominantly cytoplasmic during interphase (Figure 3C). This difference may reflect inappropriate cell cycle expression or saturation of a nuclear import mechanism. When the three major subdomains of mBub1 were assayed, only the N-terminal 331 amino acids localized to the kinetochore during prophase (Figure 3D). The central domain (amino acids 332–731) was diffusely distributed throughout the cell during both interphase and mitosis. The C-terminal kinase domain (amino acids 773–1001) also lacked kinetochore targeting ability. From this analysis, we concluded that a region in the N-terminal domain of mBub1 is sufficient for localization to the kinetochore.

Expression of Mutant mBub1 Compromises Mitotic Arrest

We reasoned that if mBub1 were required for mitotic checkpoint function, expression of a domain that localized to the kinetochore, but lacked other domains, might

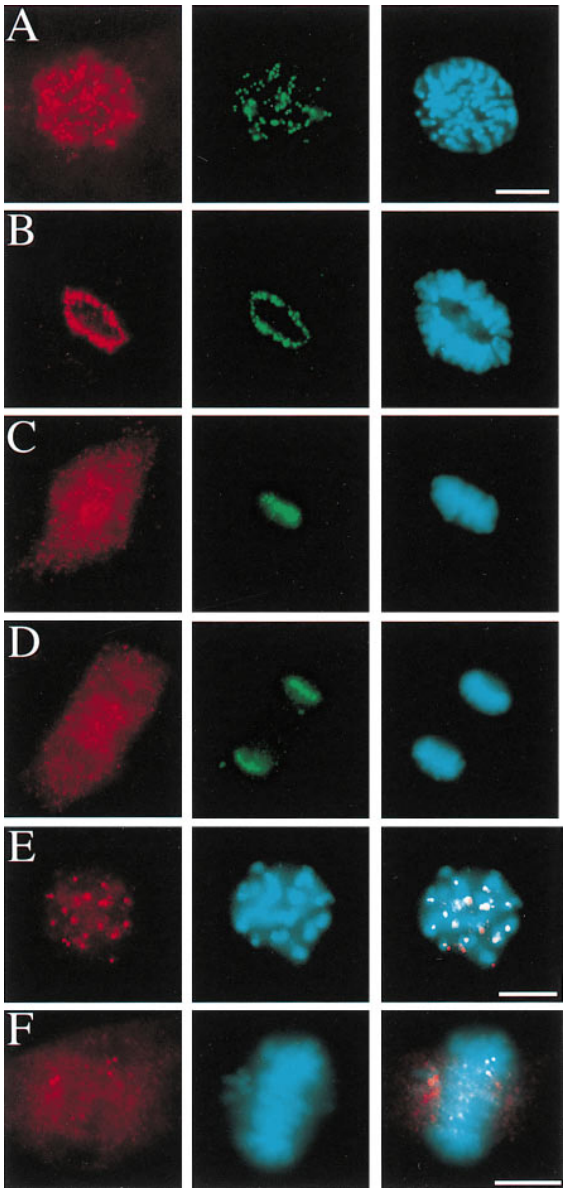

Figure 2. mBub1 Localizes to Kinetochores of Unaligned Chromosomes

Dividing mouse 10T1/2 fibroblasts (A–D) and Swiss 3T3 fibroblasts (E and F) were fixed and stained with a monoclonal antibody against murine Bub1 (red), human CREST autoimmune sera against centromere/kinetochore antigens (green), and Hoechst dye to visualize the DNA (blue).

(A) Prophase cell showing colocalization of mBub1 with CREST antigens in paired foci.

(B) Prometaphase cell showing concentration of mBub1 at kinetochores.

(C) Metaphase cell revealing loss of mBub1 staining at the kinetochore and an increase in signal throughout the cytoplasm.

(D) Anaphase cell with diffuse distribution of mBub1.

(E) In the presence of nocodazole (0.2 μg/ml), the kinetochores are unattached and display strong mBub1 staining.

(F) In the absence of nocodazole, chromosomes aligned at the metaphase plate show little mBub1 staining, while the kinetochores of a single, unaligned chromosome stain strongly.

Scale bars measure 10 μm. (B–D) are the same scale as (A).

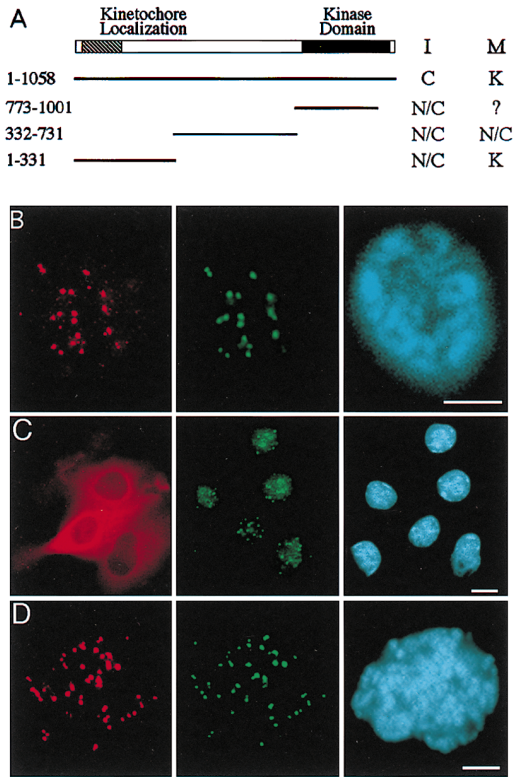

Figure 3. Kinetochore Localization Domain of mBub1

Epitope-tagged mBub1 and deletion mutants were assayed for kinetochore localization upon expression in BHK cells by immunofluorescence with the 9E10 monoclonal anti-myc antibody (left panels). Kinetochores were identified with a CREST antiserum (middle panels), and the DNA was visualized by staining with Hoechst (right panels).

(A) Schematic representation of mBub1 indicating the deletion mutants tested and their localization during interphase (I) and mitosis (M), scored as either cytoplasmic (C), nuclear (N), or kinetochore (K). The question mark (?) indicates that a mitotic cell expressing the C-terminal domain was never observed.

(B) A prophase cell expressing mBub1 showing colocalization of the Myc epitope and kinetochores.

(C) Transfected cells showing that overexpressed mBub1 is predominantly cytoplasmic during interphase.

(D) A prophase cell expressing the N-terminal domain of mBub1 (amino acids 1–331) showing its colocalization with kinetochores.

have a dominant-negative phenotype. To test this possibility, we stably transfected tTA-HeLa cells, which express a tetracycline-controlled transactivator (Gossen and Bujard, 1992), with a construct expressing a myc-tagged, N-terminal mBub1 (N-mBub1, amino acids 1–331) fusion protein under the control of a tetracycline-responsive promoter (Gossen and Bujard 1992). Two independent cell lines (H261 and H533) were characterized in detail, and both behaved in a similar manner. Data from one line, H261, are presented here. When H261 was grown in the absence of tetracycline to induce expression of the tagged N-mBub1, the myc epitope was detected at kinetochores of prophase cells (Figure 4A, Tet^{-}). Conversely, in the presence of tetracycline, no expression of N-mBub1 was detected (Figure 4A, Tet^{+}).

To assay the ability of N-mBub1 to suppress the mitotic checkpoint, cells from the H261 and the parental

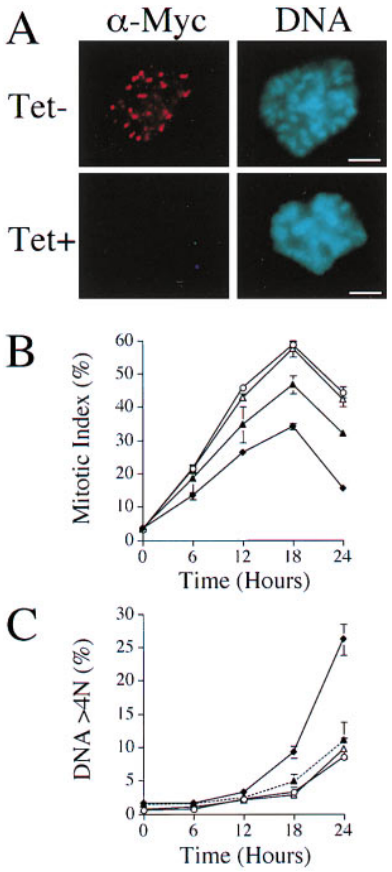

Figure 4. N Terminus of mBub1 Disrupts Mitotic Checkpoint

(A) Immunolocalization of Myc-tagged N terminus (amino acids 1–331) of mBub1 (left panel) in prophase H261 cells cultured for 48 hours in the absence (Tet^-) and presence (Tet^+) of tetracycline. DNA was stained with Hoechst dye (right panels). Expression of the N-mBub1 is only detectable in the absence of tetracycline (Tet^-).

(B and C) Flow cytometric analysis of H261 cells expressing the N terminus of mBub1. H261 cells and the parental cell line, tTA-HeLa, were cultured in the presence or absence of tetracycline for 48 hours and then treated with nocodazole (0.2 μg/ml). At the time points indicated, cells were harvested and stained with MPM-2 to determine the mitotic index and propidium iodide to determine DNA content. The percentage of cells in mitosis (B) and with DNA contents greater than 4N (C) were plotted as a function of time. Open circle, tTA-HeLa Tet^+; open triangle, tTA-HeLa Tet^-; closed triangle, H261 Tet^+; closed diamond, H261 Tet^-. Each point shows the average and standard deviation derived from three experiments.

line (tTA-HeLa) were grown in the presence (Tet^+) or absence (Tet^-) of tetracycline for 48 hours prior to spindle disruption with nocodazole. Cells were then harvested every six hours, fixed, stained with the MPM-2 monoclonal antibody (Davis et al., 1983) and propidium iodide to determine the mitotic index and DNA content, respectively, and analyzed by flow cytometry. The mitotic index of all populations increased with time in nocodazole, indicating a mitotic arrest (Figure 4B). As observed with other cell lines, this arrest is only transient (Kung et al., 1990; Andreassen and Margolis, 1994; Minn et al., 1996), and after 18 hours, cells began to exit mitosis, despite the continued presence of nocodazole. However, the rate at which Tet^- H261 cells accumulated in mitosis (34% in 18 hours) was reduced compared to both Tet^+ H261 cells (47% in 18 hours) and to the parental cell line (59% and 57% in the presence and absence of tetracycline, respectively, in 18 hours) (Figure 4B). In addition, the Tet^- H261 population accumulated cells with DNA contents greater than 4N at a faster rate than the control cell populations (Figure 4C), indicative of cell cycle progression. The most likely explanation for these results is that cells expressing the N terminus of mBub1 fail to maintain mitotic arrest in response to spindle damage, and continue cell cycle progression, despite the absence of chromosome segregation and cytokinesis. The reduced rate of accumulation of mitotic cells in the Tet^+ H261 population relative to the parental cell line is probably due to low levels of expression of N-mBub1 in the presence of tetracycline. Indeed, line H533, which appears to express less N-mBub1 in the presence of tetracycline, accumulates mitotic cells at a rate (53% in 18 hours, data not shown) more typical of the parental cell line.

Cells Expressing Dominant-Negative mBub1 Escape Cell Death

To examine the apparent suppression of the mitotic checkpoint by N-mBub1 in more detail, H261 cells were first synchronized at the G1/S boundary using a double thymidine block in the presence (Tet^+) and absence (Tet^-) of tetracycline. Following release from G1/S, nocodazole was added to prevent spindle assembly. Cells harvested at various time points were analyzed by flow cytometry to determine the mitotic index and DNA content. For the first 12 hours after release from G1/S, both populations (Tet^+ and Tet^-) progressed through S phase and entered G2 and mitosis with similar kinetics (Figures 5A and 5B). However, after 12 hours, the behavior and fates of these populations were dramatically different. The cells not expressing the N-mBub1 mutant (Tet^+) continued to accumulate in mitosis for the next six hours, and subsequently exited mitosis at approximately 18 hours (Figure 5B). Significantly, this population then accumulated cells with DNA contents less than 4N (Figure 5A, Tet^+), indicative of cell death. The cells expressing N-mBub1 (Tet^-) also continued to accumulate in mitosis up to the 18 hour time point, but the mitotic index was much reduced compared to the Tet^+ cells (Figure 5B), suggesting that many cells were already leaving mitosis. Following exit from mitosis, cells in this population accumulated DNA contents greater than 4N (Figure 5A, Tet^-), indicating continued cell cycle progression. By 42 hours, the majority of these cells had completed S phase, producing a large population of cells with an 8N DNA content. In addition, these cells entered a second mitosis (Figure 5B).

To clarify the different fates of the cells cultured with or without tetracycline, the DNA histograms, nuclear integrity, and cell morphology of cells harvested at 42 hours were compared. Of the cells in the Tet^+ population, 49% had DNA contents below 4N, while only 30% had DNA contents greater than 4N (Figure 5C). The majority of cells contained highly condensed, fragmented chromatin and showed significant membrane blebbing (Figures 5D and 5E), indicative of apoptosis. These observations are consistent with previous studies of certain cell lines, which undergo apoptosis following exit

from mitosis in the presence of nocodazole (Minn et al., 1996). In contrast, only 16% of the cells in the Tet$^-$ population appeared apoptotic, whereas 73% had DNA contents greater than 4N (Figure 5C). Moreover, these cells were large and multinucleated, consistent with an 8N DNA content and having left mitosis without completing chromosome segregation (Figures 5D and 5E).

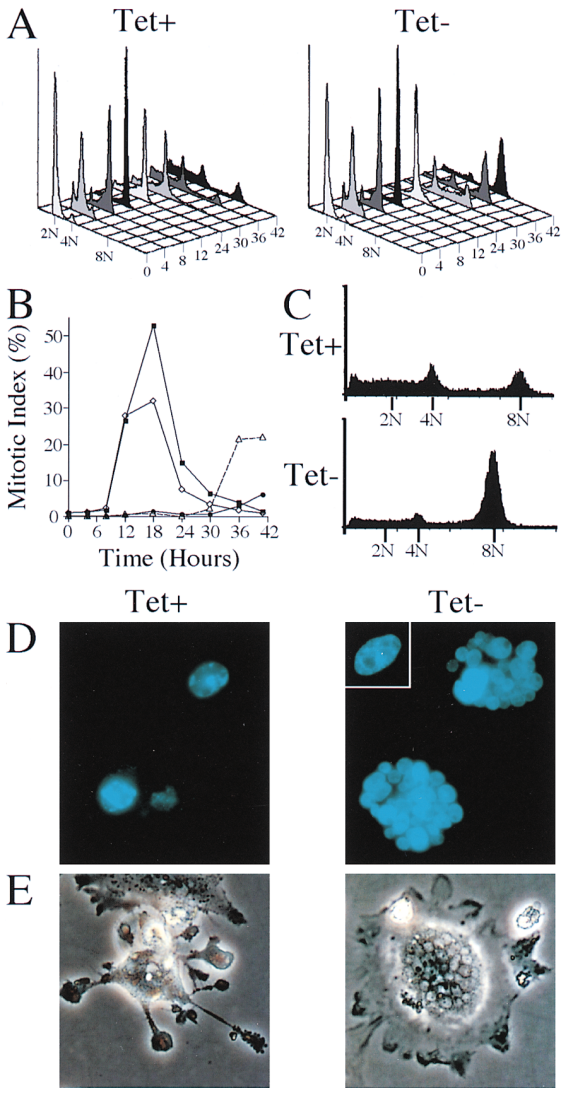

Figure 5. Dominant-Negative mBub1 Suppresses Mitotic Checkpoint and Apoptosis

H261 cells were synchronized at the G1/S boundary by a double thymidine block with (Tet$^+$) and without (Tet$^-$) tetracycline, released for six hours, and then exposed to nocodazole (0.2 μg/ml). At the times indicated, cells were stained with propidium iodide and MPM-2 and analyzed by flow cytometry to determine DNA content and mitotic index, respectively.

(A) Three-dimensional overlays of DNA histograms. The X axis represents relative DNA content and the Z axis hours after release from the second thymidine block. After 12 hours, the Tet$^+$ population accumulates cells with DNA contents below 4N, indicative of cell death. In contrast, the Tet$^-$ population accumulates cells with DNA contents greater than 4N, indicating continued cell cycle progression. Note that the 18 hour time point appears identical to the 12 hour time point and is therefore not shown.

(B) Mitotic index of cell populations as determined by MPM-2 staining. Based on propidium iodide fluorescence, the mitotic cells can be distinguished as having a DNA content of 4N or 8N, and are therefore plotted separately. Closed square, Tet$^+$, 4N; open diamond, Tet$^-$, 4N; closed circle Tet$^+$, 8N; open triangle, Tet$^-$, 8N.

(C) DNA content histograms of the Tet$^+$ and Tet$^-$ populations 42 hours after release from G1/S, highlighting the different fates of the cells following exit from mitosis in the absence of a spindle.

(D) Representative Hoechst fluorescence images of cells revealing highly condensed and fragmented nuclei in the Tet$^+$ population and intact, multinucleated cells in the Tet$^-$ population. The inset shows a normal cell for size comparison.

(E) Representative phase contrast images of living cells in the Tet$^+$ and Tet$^-$ populations. Cells in the Tet$^+$ population are rounded up with heavily blebbed membranes and indistinct nuclei. In contrast, cells in the Tet$^-$ population are relatively large, flat, and multinucleated, indicative of mitotic exit without chromosome segregation.

mBub1 Regulates Timing of a Normal Mitosis

To determine if expression of N-mBub1 affected progression through a normal mitosis, H261 cells were synchronized at the G1/S boundary in the presence and absence of tetracycline and analyzed for progression through mitosis in the absence of microtubule inhibitors. Both the Tet$^+$ and Tet$^-$ populations progressed through S phase and into G2/M with similar kinetics (Figure 6A). Eighteen hours after release from G1/S, the vast majority of cells in both populations had returned to G1, suggesting that expression of N-mBub1 did not adversely affect chromosome segregation. However, at the 12 hour time point, more cells in the Tet$^-$ population had returned to G1 relative to the Tet$^+$ population (Figure 6A). Because the Tet$^+$ and Tet$^-$ cells enter mitosis at the same time (Figure 5B), this difference must be due to an accelerated exit from mitosis by cells in the Tet$^-$ population. To further quantitate this observation, the experiment was repeated taking time points every hour. The difference between the times taken for half of the cells to return to G1 was then estimated (Figure 6B). Based on four such experiments, this analysis indicates that the Tet$^-$ cells returned to G1 approximately 25 minutes sooner than the cells in the Tet$^+$ population.

Discussion

We have shown that murine Bub1 localizes to the kinetochore and is not only essential for cell cycle arrest following spindle damage in mammalian cells, but also determines the time allowed chromosomes to align on a normal spindle. In addition, we have found that the same cell line can exhibit radically different responses upon exit from mitosis in the absence of a spindle: cells that breach an activated mitotic checkpoint undergo apoptosis, while those that pass through a suppressed checkpoint advance into additional cell cycles. These observations suggest that the apoptotic response is not to polyploidy or aberrant chromosome segregation, as these exist in both cases, but rather to functional links between the exit from mitosis and a G1 checkpoint.

mBub1, a Kinase at the Kinetochore

Elegant experiments in higher eukaryotes predict that a tension-sensitive kinase at the kinetochore regulates

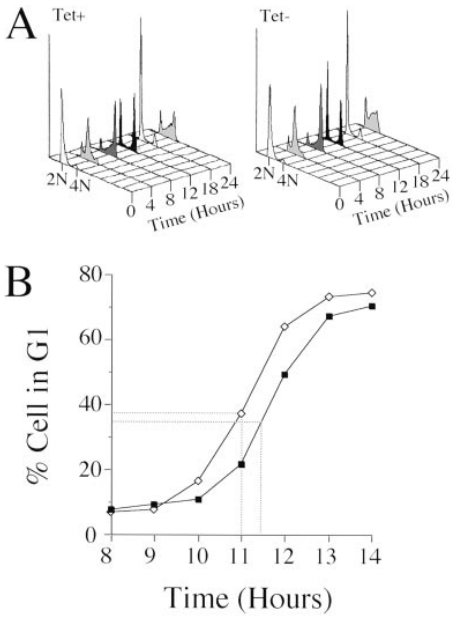

Figure 6. mBub1 Regulates the Exit from a Normal Mitosis

(A) Three dimensional overlays of DNA histograms of Tet^+ and Tet^- H261 populations at progressive time points following release from a G1/S block. In the absence of nocodazole, cells in the Tet^- population return to G1 sooner than cells in the Tet^+ population.

(B) Quantitation of cells in G1 following release from the G1/S boundary indicates that cells in the Tet^- (open diamond) population return to G1 approximately 25 minutes before cells in the Tet^+ (closed square) population.

the onset of anaphase (Campbell and Gorbsky, 1995; Li and Nicklas, 1995; Nicklas et al., 1995; Nicklas, 1997). Bub1 has several of the properties expected of this hypothetical sensor. First, catalytically inactive alleles of yeast *BUB1* fail to complement *BUB1* deletion strains, indicating that Bub1 kinase activity is essential for checkpoint function (Roberts et al., 1994). Second, mBub1 associates with kinetochores of unaligned chromosomes, and this localization is required for checkpoint function. Interestingly, kinetochore localization of mBub1, like both XMad2 and the 3F3 epitope (Gorbsky and Ricketts, 1993; Nicklas et al., 1995; Chen et al., 1996), appears to be controlled by local events at the kinetochore (Figure 2F). It is possible that upon correct attachment, the conformation of the kinetochore is altered such that Bub1 dissociates, thus relieving the checkpoint. Alternatively, attachment could first suppress Bub1 kinase activity to rapidly alleviate the checkpoint. Subsequent dissociation of Bub1 may then reduce the chance of reactivating the checkpoint during chromosome movements that follow correct alignment (Nicklas et al., 1995). Whether Bub1 is responding directly to a spindle event, such as tension or microtubule attachment, or is downstream of another kinase that senses spindle events, is not known. While genetic studies in yeast indicate that the checkpoint signaling pathway is complex (Elledge, 1996; Hardwick et al., 1996), they indicate that Bub1 is at or near the top of this transduction cascade, consistent with the possibility that Bub1 responds directly to a spindle event. Experiments to address how Bub1 kinase activity and localization are regulated will undoubtedly shed light on the mechanisms of tension transduction at the kinetochore.

mBub1 Regulates Normal Mitotic Timing

A fundamental question concerning all checkpoints is whether they function during every cell cycle or only during abnormal situations, such as DNA damage or loss of spindle integrity. We addressed this question by conditional expression of a dominant-negative mBub1, which did not effect progression through S phase, G2, or entry into mitosis. Moreover, it did not prevent chromosome segregation in the absence of nocodazole, although it did compromise the ability of cells to maintain arrest in response to spindle damage. However, in the presence of a functional spindle, cells expressing dominant-negative mBub1 left mitosis approximately 25 minutes before control cells (Figure 6), indicating that Bub1 regulates progression through a normal mitosis. Despite this accelerated mitosis, the DNA profiles of the resulting G1 populations were apparently normal, suggesting that the chromosomes still aligned prior to anaphase. To explain these observations, we present a model (Figure 7) in which the dominant-negative mBub1 competes with endogenous Bub1 for kinetochore binding sites, thus reducing the amount of functional Bub1 at the kinetochore. (Note that we have not been able to determine this directly in Tet^- H261 or transfected BHK cells because the monoclonal antibodies against murine Bub1 cross-react very poorly with human or hamster Bub1.) This lowers the maximum strength of the mitotic checkpoint signal toward a threshold, below which the anaphase-promoting complex (APC) is activated (King et al., 1995; Irniger et al., 1995; Sudakin et al., 1995; Tugendreich et al., 1995; Minshull et al., 1996; Zachariae and Nasmyth, 1996) and exit from mitosis is initiated by cyclin degradation. This model also draws upon data from an analysis of mitotic PtK_1 cells, which revealed the mean time from nuclear envelope breakdown (NEB) to anaphase to be approximately 50 minutes (Rieder et al., 1994). The prometaphase time window, during which chromosome alignment took place, was 27 minutes. In addition, this analysis revealed a 23 minute delay between alignment of the last chromosome and anaphase onset (Figure 7A). During prometaphase, the strength of the Bub1 checkpoint signal probably decreases as kinetochores attach to the spindle until, upon alignment of the last chromosome, it falls below the threshold required to prevent anaphase (Figure 7B, −nocodazole). Because the dominant-negative mBub1 lowers the checkpoint signal toward this threshold, the prometaphase time window is reduced and, hence, exit from mitosis is accelerated by about 25 minutes (Figures 7A and 7B). However, the 23 minute delay prior to anaphase is probably still sufficient for most chromosomes to align (Figures 7A and 7B). Over several generations, however, a reduced Bub1-dependent prometaphase period would probably increase the frequency of chromosome missegregation events. Indeed, vegetative growth of yeast *Bub1* mutants is slow, suggesting an accumulation of defects in the absence of this checkpoint (Hoyt

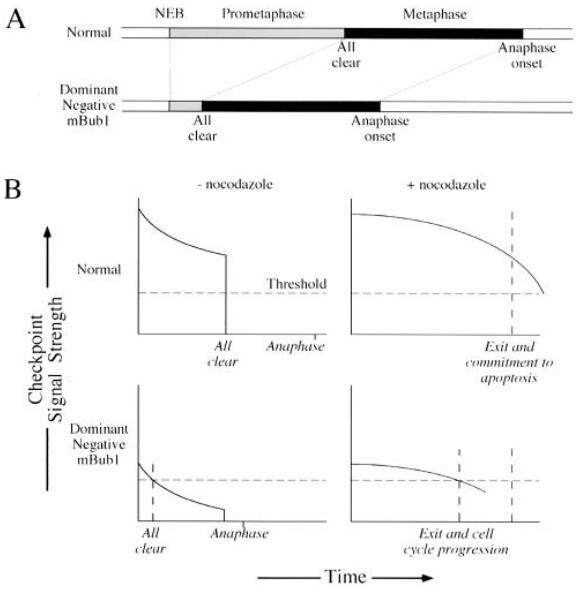

Figure 7. Pathways to Exit Mitosis

(A) A temporal scheme of progression through a normal mitosis (upper panel) and when the mitotic checkpoint is compromised (lower panel). After nuclear envelope breakdown (NEB), the mitotic checkpoint maintains a time window (lightly shaded box) that allows chromosome alignment. After the last chromosome aligns, the "all clear" signal is given and, after a fixed time (darkly shaded box), the cell enters anaphase. When the checkpoint is compromised, the time window closes soon after entry into mitosis, prematurely giving the all clear signal. The time delay before anaphase onset is unaltered.

(B) Hypothetical graphs indicating how mitotic checkpoint signal strength (vertical axis) varies over time (horizontal axis) during mitosis. The threshold is the strength of the checkpoint signal required to prevent cyclin degradation. If the mitotic checkpoint is fully functional, the initial signal strength is high and, in the absence of nocodazole, it is maintained above the threshold until the last chromosome attaches. In cells expressing dominant-negative mBub1, the initial signal strength is low and quickly falls below the threshold, despite the presence of unattached chromosomes. Because of the fixed delay between the all clear signal and the onset of anaphase, there is still sufficient time for chromosome alignment. In the presence of nocodazole, the signal strength is maintained at a high level but decays slowly over time. If the checkpoint is still active when the cells exit mitosis, then apoptosis occurs. However, if the checkpoint is compromised, the initial signal strength is low and falls below the threshold much sooner, resulting in normal mitotic exit and cell cycle progression.

et al., 1991). Overall, these data support the notion that Bub1 not only functions in response to spindle damage, but monitors the completion of the normal interactions between kinetochores and microtubules during prometaphase.

Pathways to Exit Mitosis: Life versus Death

The model also helps to explain the different cell fates following exit from mitosis with a damaged spindle. In the absence of a spindle, kinetochores are unattached, and hence the strength of the Bub1 checkpoint signal is initially at a maximum (Figure 7B, +nocodazole). However, over time, the signal probably decays, eventually falling below the threshold, resulting in exit from mitosis. Expression of the dominant-negative mBub1 reduces the initial signal, and hence exit from mitosis occurs sooner. Exit from mitosis in this manner produces cells that are then competent to enter additional cell cycles (Figure 5; Kung et al., 1990; Andreassen and Margolis, 1994). However, exit from mitosis with a damaged spindle can lead to apoptosis (Figure 5; Minn et al., 1996), which may provide a solution to the potential risk of aneuploidy. The molecular mechanisms that activate apoptosis in response to aberrant mitosis are not known. It has been suggested that cells may monitor some structural feature, such as aneuploidy (Rudner and Murray, 1996), DNA damage, topological abnormalities, unsegregated chromatids, or the presence of multiple centrosomes (Minn et al., 1996). However, the data presented here argue against the notion that apoptosis is induced by a structural aspect of an aberrant mitosis. Our analysis of cells that leave mitosis with a damaged spindle indicates that their subsequent fate depends on the status of the mitotic checkpoint: exit from mitosis with a functional checkpoint resulted in cell death, whereas exit from mitosis with a compromised checkpoint resulted in cell cycle progression (Figure 5). Therefore, the manner in which cells exit mitosis must somehow signal the apoptotic response in G1. Clues to such signals may come from recent evidence showing that cells can inactivate MPF, and thus exit mitosis, not by targeting cyclin B for degradation, but by inhibitory phosphorylation of cdc2 (Minshull et al., 1996; Wang and Burke, 1996). This novel exit pathway may play a role in adaptation to prolonged mitotic arrest in yeast (Minshull et al., 1996; Rudner and Murray, 1996). It is possible that this adaptive pathway is conserved in mammalian cells and that mitotic exit via this pathway may directly activate apoptosis in the following G1 (Figure 7B). Alternatively, if cells exit mitosis prematurely, either by cyclin degradation or another pathway, without first completing kinetochore attachment, the Bub1-dependent checkpoint signal may still be active. The presence of an active mitotic checkpoint signal in G1 may alert the cell of an aberrant mitosis and thus trigger the apoptotic response (Figure 7B). These data suggest that functional links between the exit from mitosis and subsequent G1 checkpoints exist, possibly to protect multicellular organisms from the consequences of checkpoint failure.

Experimental Procedures

Cell Culture

All cell lines (baby hamster kidney [BHK], human tTA-HeLa, mouse 10T1/2 and 3T3 fibroblasts, mouse NOS myeloma cells, and mouse hybridomas) were cultured in Dulbecco's modified Eagle's media (DMEM) supplemented with 10% fetal calf serum (FCS; Hyclone), 2 mM glutamine, penicillin, and streptomycin. tTA-HeLa cells were cultured in the presence of 100 μg/ml geneticin and 2 μg/ml tetracycline (Bio-Quant). For monoclonal antibody production, fusions were plated in DMEM supplemented with 20% serum (Sigma CPSR-3), 5% NCTC, 1% NEAA, 1% glutamine, 1% pen/strep, and 1x HAT. Poorly growing hybridomas were grown on gamma-irradiated 3T3 mouse fibroblast feeder layers. All tissue culture reagents were from GIBCO BRL unless otherwise stated.

DNA Manipulations

A partial mouse cDNA sequence (A005, GenBank accession number L26607; Kerr et al., 1994) encoding an open reading frame with

homology to ScBub1 was identified from a search of the NCBI dbEST using the BLAST algorithm (Altschul et al., 1990). Two oligonucleotide primers (A005.1, 5′ ATC ATT CAT GGA GAC ATT AAG CCA GAT 3′; and A005.2, 5′ CAT TAG TTA GAC CAT CAA GGT ACC GAA 3′) were used to PCR amplify a corresponding 224 bp fragment from a mouse teratocarcinoma cDNA library (Stratagene). This fragment was radiolabeled and used to probe phage from the same cDNA library (Sambrook et al., 1989). cDNA inserts from positive phage were subcloned into pBS SK− (Stratagene) and sequenced on both strands.

Mammalian Expression Constructs

The mammalian expression vectors used for transient transfections were based on pcDNA-3 (Invitrogen) modified to include the 5′ untranslated sequence from the human lamin A cDNA and N-terminal Myc or Myc-GFP epitope tags (Shibasaki et al., 1996). The constructs shown in Figure 3 were generated by either direct subcloning of phage inserts or PCR amplification using Pfu polymerase (Stratagene). To generate the tetracycline-responsive expression construct, the XhoI site in pUHD 10-3 (Gossen and Bujard, 1992) was first destroyed by digestion with XhoI, followed by Klenow repair and religation. The polylinker was then modified by digestion with SacII and BamHI and the ligation of a synthetic oligonucleotide polylinker containing the restriction sites for EcoRI/SacII/XhoI/BamHI/NotI/ApaI. A fragment containing the 5′ lamin A untranslated region and the Myc tag epitope was then cloned into the EcoRI/XhoI sites to create pUHD Myc. The N-terminal domain of mBub1 encoding amino acids 1–331 was then cloned into the XhoI/NotI sites to create pUHD N-mBub1.

Monoclonal Antibody Production

The central portion of mBUB1 encoding amino acids 332–731 was cloned as a BamHI/NotI fragment into pGEX-4T-3 (Pharmacia) and transformed into the E. coli strain BL21. The GST fusion protein was purified from bacterial lysates by binding to glutathione Sepharose beads (Pharmacia). Immunizations and fusions were done essentially as described (Harlow and Lane, 1988). Ten days post fusion, hybridoma supernatants were screened for antibodies against the central domain of mBUB1 by an ELISA assay (Pierce) and immunofluorescense of BHK cells transfected with the central domain (see below). Two hybridomas producing antibodies of interest, 4B12 and 3F4, were subcloned and expanded.

Immunofluorescence

The following antibodies were used: 4B12 (anti-mBUB1) mouse monoclonal tissue culture supernatant diluted 1:2; human autoimmune CREST sera (anti-centromere/kinetochore) diluted 1:2000; 9E10 (anti-Myc) mouse monoclonal purified from tissue culture supernatant, diluted 1:1000; Cy3-conjugated donkey anti-mouse (Cy3 DaM) at 1:1000; FITC-conjugated donkey anti-mouse (FITC DaM) at 1:500; Cy3-conjugated donkey anti-human (Cy3 DaH) at 1:1000; and FITC-conjugated donkey anti-human (FITC DaH) at 1:500. All secondary antibodies were from Jackson Immunoresearch Laboratories. Cell fixation and immunofluorescence were performed essentially as described (Heald et al., 1993).

Transfections and Generation of Stable Cell Lines

BHK and tTA-HeLa cells were transiently transfected by the calcium phosphate method essentially as described (Heald et al., 1993). To generate tetracycline-responsive cell lines, tTA-HeLa cells were cultured in the presence of tetracycline on 18 mm coverslips and cotransfected with PvuI-digested pUHD N-mBub1 and pPUR (Clontech) digested with EcoRI, at a ratio of 20:1. Parallel transfections were performed in the absence of tetracycline and analyzed by immunofluorescence to monitor transfection efficiency. Transfected cells were selected in 1 μg/ml puromycin, individual colonies picked, expanded, and then screened by immunofluorescence for expression of N-mBUB1 in the absence of tetracycline. Two independent cell lines, H261 and H533, were characterized further.

Cell Cycle Analysis

Approximately 0.5×10^6 HeLa cells were plated in 10 cm dishes in the presence and absence of tetracycline. After 48 hours, nocodazole (Sigma) was added to a final concentration of 0.2 μg/ml. At the times indicated, cells were harvested and analyzed as described below. To generate synchronized populations, H261 cells were first arrested at the G1/S boundary by a double thymidine block. At various time points after release, cells were harvested and analyzed. Where indicated, nocodazole was added to a final concentration of 0.2 μg/ml. For flow cytometric analysis, loosely attached cells were first collected and then pooled with attached cells removed by trypsinization. Following fixation in 70% ethanol, the cells were washed in PBS and then incubated with the MPM-2 monoclonal antibody (Davis et al., 1983) diluted 1:750 in PTB (1× PBS, 0.05% Tween-20, 5% BSA) for 1 hour at 4°C. The cells were then washed twice for 5 minutes in PBS and incubated with FITC DaM diluted 1:100 in PTB for 1 hour at 4°C. Following two 5 minute washes in PBS, the cells were stained with propidium iodide (40 μg/ml final concentration) and treated with RNase A (50 μg/ml) for 30 minutes at room temperature. Samples of 10,000 cells were then analyzed on a Becton Dickinson FACScan and data presented using the LYSYS II software.

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